Fusobacterium nucleatum subsp. *fusiforme* subsp. nov. and *Fusobacterium nucleatum* subsp. *animalis* subsp. nov. as Additional Subspecies within *Fusobacterium nucleatum*

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Using a variety of physiological, biochemical, and molecular systematic analyses, we have shown previously that there are four groups within the species *Fusobacterium nucleatum*. Two of these groups of strains correspond to the recently proposed taxa *F. nucleatum* subsp. *nucleatum* and *F. nucleatum* subsp. *polymorphum*. In this paper we show that the two remaining groups are distinct and formally propose that they should be recognized as *F. nucleatum* subsp. *fusiforme* (type strain, NCTC 11326) and *F. nucleatum* subsp. *animalis* (type strain, NCTC 12276). The tests which we used did not allow a full assessment of the status of *F. nucleatum* subsp. *vincentii* compared with *F. nucleatum* subsp. *nucleatum*.

Fusobacterium nucleatum, the type species of the genus *Fusobacterium*, has been shown to comprise a heterogeneous collection of strains (1, 3, 4). Recently, Dzink et al. (2) described three subspecies, *F. nucleatum* subsp. *nucleatum*, *F. nucleatum* subsp. *polymorphum*, *F. nucleatum* subsp. *vincentii*, while almost simultaneously we described four subspecies (5) following a series of detailed systematic analyses (3–6, 8). The descriptions of *F. nucleatum* subsp. *nucleatum* and *F. nucleatum* subsp. *polymorphum* in two previous studies (2, 5) appear to be consistent, and the same type strains and the same names were proposed independently for each subspecies. It is evident from a previous description of “*F. nucleatum* subsp. *animalis*” (5) that this organism is a distinct taxon within the species *F. nucleatum*. However, the taxonomic position of “*F. nucleatum* subsp. *fusiforme*” (5) remains unclear. Because of the paucity of tests used for describing *F. nucleatum* subsp. *vincentii*, it is uncertain whether this subspecies and *F. nucleatum* subsp. *fusiforme* are synonymous. In this study, we compared strains of *F. nucleatum* subsp. *fusiforme* with the type strain of *F. nucleatum* subsp. *vincentii* by using enzymic electrophoretic patterns, which have been shown previously to have significant intraspecies diagnostic value in the genus *Fusobacterium*. In addition, in this paper we formally propose recognition of *F. nucleatum* subsp. *animalis*. Our proposals are consistent with our recent rRNA sequence data (9).

MATERIALS AND METHODS

Bacterial strains. *F. nucleatum* reference strains ATCC 25586^T (T = type strain), ATCC 49256^T, ATCC 10953^T, NCTC 11326^T, and NCTC 12276^T and 10 clinical isolates resembling strains NCTC 11326^T and NCTC 12276^T were used in this study.

Growth conditions and identification. All strains were maintained by weekly subculturing on blood agar culture medium containing 5% (vol/vol) sheep blood (Oxoid) in an

atmosphere containing 10% CO₂ and 10% H₂ in N₂ at 37°C. Clinical isolates were identified as described previously (4, 7).

Electrophoresis and staining of enzymes. Cell extracts were prepared and electrophoresis was carried out on cellogel support strips as described previously (3). Glutamate dehydrogenase (GDH) and 2-oxoglutarate reductase (OGR) bands were stained as described previously (3, 4).

RESULTS AND DISCUSSION

All of the isolates were identified as members of *F. nucleatum* by their ability to produce indole, their absence of urease activity, and their ability to reduce nitrate. Butyrate and acetate were major acidic end products of metabolism. Six of the clinical isolates which we tested morphologically resembled strain NCTC 11326^T (previously designated *F. nucleatum* subsp. *fusiforme*); the electrophoretic mobilities for GDH and OGR for these strains were 1.8 and 2.0 cm, respectively. The isolates that resembled strain NCTC 12276^T (designated *F. nucleatum* subsp. *animalis*) grew in the presence of 20% bile and had both GDH and OGR activities (electrophoretic mobilities, 3.2 and 3.8 cm, respectively). Figure 1 shows the GDH electrophoretic mobilities for the five reference strains representing the subspecies examined in this study. *F. nucleatum* subsp. *polymorphum* strains had a distinct electrophoretic mobility, whereas the GDH of *F. nucleatum* subsp. *vincentii* coelectrophoresed with *F. nucleatum* subsp. *nucleatum* GDH. The OGR of *F. nucleatum* subsp. *nucleatum* and the OGR of the type strain of *F. nucleatum* subsp. *vincentii* also comigrated (Fig. 1).

In this study we could not distinguish between strains of *F. nucleatum* subsp. *nucleatum* and the type strain of *F. nucleatum* subsp. *vincentii*. This could indicate that these two subspecies are synonymous, that an incorrect reference strain of *F. nucleatum* subsp. *vincentii* has been deposited in a culture collection, or that the tests which we used cannot be used to differentiate these taxa. However, it is clear that *F. nucleatum* subsp. *vincentii* and *F. nucleatum* subsp. *fusiforme* are not synonymous as is sometimes stated (12).

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FIG. 1. GDH electrophoretic mobilities of *F. nucleatum* subsp. *fusiforme* (lane 1), *F. nucleatum* subsp. *polymorphum* (lane 2), *F. nucleatum* subsp. *nucleatum* (lane 3), *F. nucleatum* subsp. *animalis* (lane 4), and *F. nucleatum* subsp. *vincentii* (lane 5).

The strains assigned to *F. nucleatum* subsp. *animalis* had the fastest-migrating enzymes on electropherograms and formed a distinct cluster that was readily recognizable from the other subspecies. When both GDH and OGR electrophoretic patterns were used as a basis for comparison, the strains assigned to *F. nucleatum* subsp. *fusiforme* (including strain NCTC 11326¹) were clearly different from *F. nucleatum* subsp. *vincentii* ATCC 49256; thus, separate subspecies status for *F. nucleatum* subsp. *fusiforme* was justified.

In previous studies we reported the absence of any clear physiological tests that can be used to differentiate among the subspecies of *F. nucleatum*. Furthermore, the levels of DNA-DNA homology when experiments are carried out under optimal hybridization conditions are high (4). Under stringent hybridization conditions, however, four centers of variations are clearly evident; these correlate with the groups formed on the basis of the GDH and OGR electrophoretic patterns (4). These groups have also been substantiated by rRNA gene restriction patterns, using a plasmid (pK3535) to probe chromosomal DNA digests of the subspecies (8). More recently, we found high levels of rRNA sequence similarity (ca. 97.3 to 98.4%) that are consistent with a single species. However, an unrooted phylogenetic tree based on K_{nuc} (evolutionary distance) values clearly delineated the four taxa. Therefore, we formally propose that *F. nucleatum* subsp. *fusiforme* and *F. nucleatum* subsp. *animalis*, which until now have been unclassified, should be recognized as new subspecies of *F. nucleatum*. Descriptions of these two taxa are given below.

Description of *Fusobacterium nucleatum* subsp. *fusiforme* subsp. nov., nom. rev., comb. nov. *Fusobacterium nuclea-*

tum subsp. *fusiforme* (fu.si.for'me. L.n. *fusiforme*, a spindle; L.n. *forma*, shape; M.L. adj. *fusiforme*, spindle shaped). Gram negative. Anaerobic. Cells are rod shaped and non-motile, do not form filaments, and are 8 to 16 μ m long by 0.5 μ m in diameter. Colonies on blood agar after 24 h are granular with very irregular edges, 10 to 13 μ m in diameter, and low cone shaped, and pitting is frequently observed if colonies are dislodged. Trypticase, protease peptone, and Bacto Casitone (Difco) markedly enhance growth in liquid medium. Few amino acids (mainly glutamate, histidine, cysteine, and serine) are utilized. The terminal pH in a glucose medium is higher than 7.0. The electrophoretic mobilities of GDH and OGR are 1.8 and 2 cm, respectively. Moderate levels of H_2S are produced in liquid medium. All strains possess the constitutive enzymes leucine aminopeptidase and gamma-glutamyl aminopeptidase. Serine-valine aminopeptidase and proline aminopeptidase are not produced. Glycosidases are absent.

The cell wall peptidoglycan contains *meso*-lanthionine as its diamino acid. Possesses straight-chain, monounsaturated, and hydroxylated long-chain fatty acids but not methyl-branched acids. Tetradecanoic acid ($C_{14:0}$; ca. 24 to 30%) and hexadecanoic acid ($C_{16:0}$; 20 to 26%) are the predominant nonhydroxylated acids. The major hydroxylated fatty acids are 3-hydroxytetradecanoic acid (3OH- $C_{14:0}$; ca. 12 to 18%) and 3-hydroxyhexadecanoic acid (3OH- $C_{16:0}$; ca. 4 to 7%). The DNA base composition is 26 to 28 mol% G+C, as determined by the thermal denaturation method. The type strain is strain NCTC 11326.

Description of *Fusobacterium nucleatum* subsp. *animalis* subsp. nov. *Fusobacterium nucleatum* subsp. *animalis* (a.ni.ma'lis. L. n. *animal*, animal; *animalis*, of an animal). Gram negative. Anaerobic. Cells are nonmotile rods that occasionally form filaments which are up to 25 μ m long. Colonies on blood agar are round, smooth, glistening, convex, translucent white, and 1.0 to 2.0 μ m in diameter. Growth is markedly affected by the addition of Trypticase, protease peptone, and Bacto Casitone (Difco). Few amino acids (mainly histidine, arginine, cysteine, and, to a lesser extent, glutamate) are utilized. The terminal pH in a glucose liquid medium ranges between 6.8 and 7.0. Both GDH and OGR are present and exhibit fast electrophoretic mobilities (3.2 and 3.8 cm, respectively). Low levels of H_2S are produced, and growth is not affected by the addition of 20% bile. Strains are morphologically similar to *F. nucleatum* subsp. *polymorphum*, but the level of DNA-DNA homology between these two subspecies ranges between 60 and 67%. All strains possess the constitutive enzymes leucine aminopeptidase and gamma-glutamyl aminopeptidase. Serine-valine aminopeptidase and proline aminopeptidase are not produced. Glycosidases are absent.

The cell wall peptidoglycan contains *meso*-lanthionine as its diamino acid. Possesses straight-chain monounsaturated, and hydroxylated long-chain fatty acids but not methyl-branched acids. Tetradecanoic acid ($C_{14:0}$; ca. 24 to 30%) and hexadecanoic acid ($C_{16:0}$; 20 to 26%) are the major nonhydroxylated acids. The major hydroxylated fatty acids are 3-hydroxytetradecanoic acid (3OH- $C_{14:0}$; ca. 12 to 18%) and 3-hydroxyhexadecanoic acid (3OH- $C_{16:0}$; ca. 4 to 7%). The DNA base composition is 26 to 28 mol% G+C, as determined by the thermal denaturation method. The type strain is strain NCTC 12276.

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